Erythroid Cell Differentiation

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I. Introduction

The study of erythroid cell differentiation is pertinent to the study of human leukemia from two points of view. First, since a common stem cell gives rise to both erythroid and myeloid cells, it is not unexpected that disorders of myeloid cell proliferation and differentiation should be occasionally associated with abnormalities of erythroid cell differentiation. In fact in many cases of human leukemia, there are abnormalities of erythroid cells. Secondly, the study of erythroid cell differentiation can serve as an experimental model for the study of normal and abnormal gene expression, a topic of vital importance to the understanding of the etiology and pathogenesis of human leukemia. The erythoid cell provides a number of advantages as a model system for the study of the control of gene expression. This highly specialized cell devotes approximately 95 % of its protein synthesis to the production of one protein, hemoglobin, and therefore only a limited number of the cell's genes are expressed. In addition, a number of biochemical techniques are currently available for the isolation, characterization and quantitation of globin messenger RNA (mRNA), the necessary intermediary between globin gene expression and globin chain synthesis.

Erythroid cell differentiation can be considered from two points of view: 1) differences between fetal and adult mature red blood cells; and 2) differences between erythroid cells at different stages of morphologic maturation. We will discuss first the abnormalities of red cell differentiation, mainly the emergence of fetal erythropoiesis, which can occur during the course of various human leukemias. Then we will discuss experimental studies on the quantitation of heme synthesis, globin synthesis and globin messenger RNA content in murine erythroid cells at different stages of maturation.

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Abbreviations used:
Hb: hemoglobin; mRNA: messenger RNA.
RNase: ribonuclease.
cDNA: DNA copy of globin mRNA synthesized by RNA dependent DNA polymerase (reverse transcriptase) of avian myeloblastosis virus.

II. Abnormalities of erythroid cells in the human leukemias

1. Abnormal proliferation of erythroid cells.

A number of abnormalities of erythroid cell proliferation and differentiation have been observed in various human leukemias. Some of these abnormalities can be traced to the fact that a common stem cell gives rise to all three types of blood cells: granulocytes, erythroid cells and platelets. In the myeloproliferative disorder, polycythemia vera, which can be considered as a "pre-leukemic" condition, there must be autonomous proliferation of the common progenitor stem cell, because all three cell lines proliferate and accumulate in excess with resulting erythrocytosis, granulocytosis, and thrombocytosis. After many years this condition can revert to myelofibrosis with myeloid metaplasia and/or acute myelogenous leukemia; very rarely Philadelphia chromosome-positive chronic myelogenous leukemia may develop after a long period of myelofibrosis and myeloid metaplasia and prior to acute myeloblastic transformation.

In chronic myelogenous leukemia, the acquired chromosomal marker in the leukemic myeloid cells, the Philadelphia or Ph_1 chromosome is present not only in the granulocytes but also in the erythroid (and megakaryocytic) precursor cells. This finding again points to a lesion in the common progenitor cells as a basis for at least some forms of leukemia.

Since the common stem cell is affected in at least some myeloid leukemias, it is not unexpected that in some forms of myeloid or undifferentiated leukemia, there appears to be an associated frank neoplastic transformation of the erythroid cell line: thus the term erythroleukemia, also referred to as the DiGuglielmo syndrome. In this condition there are bizarre, multinucleated megaloblastic erythroid precursors, clover leaf nuclei, abnormal mitoses with endoreduplication, and bizarre mature red cell morphology; almost invariably there is concomitant or subsequent proliferation of myeloblasts and development of frank acute myeloblastic leukemia.

2. Abnormal hemoglobin synthesis

In certain cases of erythroleukemia and more rarely in other myeloproliferative disorders, an abnormality of hemoglobin synthesis has been detected, termed acquired hemoglobin H (Hb H) disease. Normal hemoglobin consists of two α and two β chains ($\alpha_2\beta_2$), and normally there is equal synthesis and accumulation of α and β chains in erythroid cells. If there is decreased synthesis of α chains relative to β chains, then β chains will accumulate in excess and form tetramers of Hb H (β_4). Hb H is relatively unstable or insoluble: with time it precipitates in the cell, forming inclusion bodies which damage the red cell membrane and lead to premature destruction of the red cell. Studies of globin chain synthesis have been reported in one such case of acquired Hb H disease and these studies directly demonstrated a decrease in α chain synthesis relative to β chain synthesis (1). Other studies have indicated that the defect, in at least the case studied, is a clonal one, and limited to only some but not all of the patient's red cells (?the neoplastic clone) (2).

Hb H disease more commonly occurs as an inherited disorder, a form of α-thalassemia in which there is a genetic defect causing reduction of α chain syn-

thesis. In this latter condition, the patient has no increased susceptibility to developing leukemia.

3. Fetal hemoglobin synthesis

During human development there is a change in hemoglobin synthesis from fetal hemoglobin synthesis (Hb F: $\alpha_2\gamma_2$) to adult hemoglobin synthesis (Hb A: $\alpha_2\beta_2$) [Fig. 1]. The phenomenon starts during the third trimester of pregnancy and is

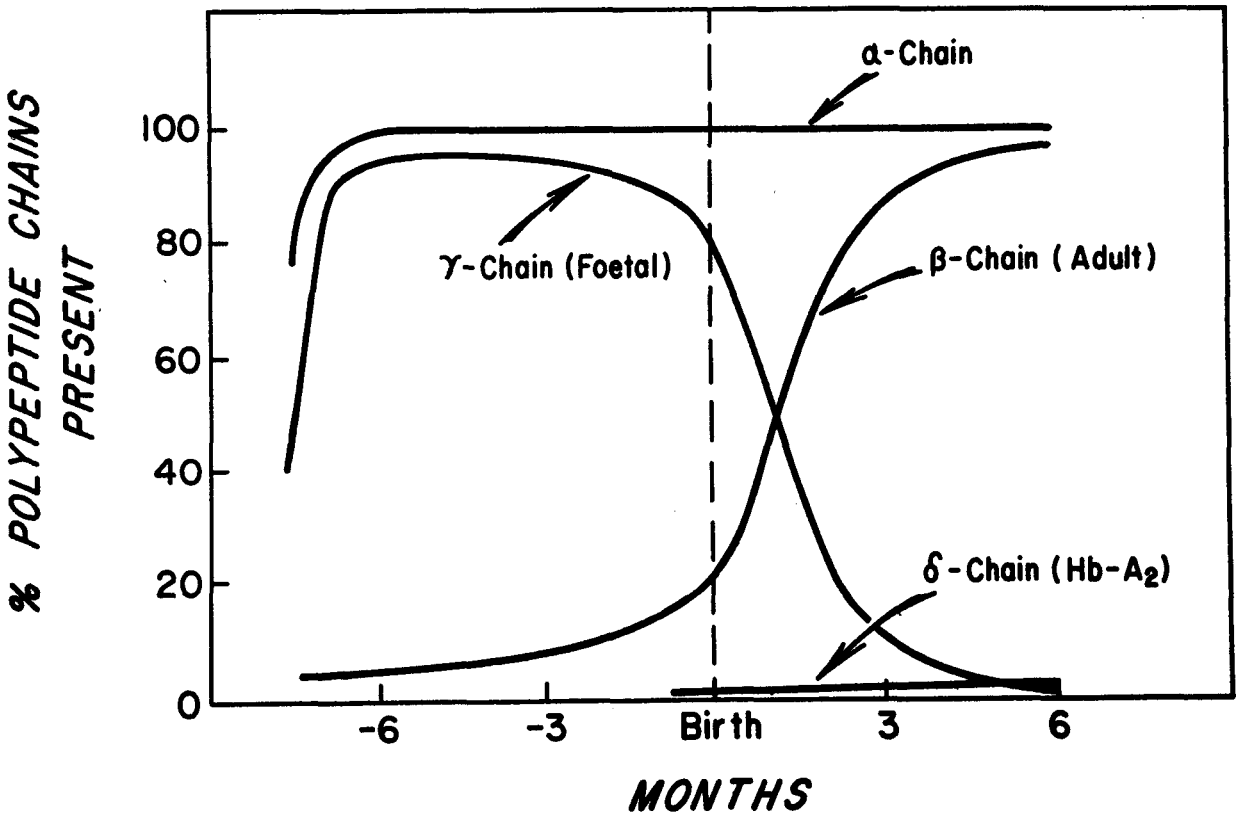

Fig. 1: Diagrammatic representation of the changes in human globin synthesis during prenatal and neonatal development. (Modified from Huehns *et al,* Cold Spring Harbor Symp. Quant. Biol. (1964) *19*, 327).

usually complete by 6 months of age. The process involves the inactivation of the genes for the γ globin chains and the activation of the genes for the β globin chains. The precise mechanism involved and the factors controlling it are unknown. There are two different types of γ chains of Hb F, which are the products of two different genes. The two γ chains differ by only one amino acid residue at position 136 of their amino acid sequence: in one chain it is alanine (the $^{A}\gamma$ chain), in the other it is glycine (the $^{G}\gamma$ chain). The relative amounts of $^{A}\gamma$ and $^{G}\gamma$ chains produced vary during development: in the fetus and newborn there is more $^{G}\gamma$ than $^{A}\gamma$ synthesis, but in the adult the small amount of residual γ chain synthesis consists of more $^{A}\gamma$ chain synthesis than $^{G}\gamma$ chain synthesis (Table 1). There are also other differences between fetal and adult red blood cells (Table 1): in fetal cells there is virtual absence of Hb A_2 ($\alpha_2\,\delta_2$) and of the enzyme carbonic anhydrase B, whereas these proteins are easily detected in hemolysates of adult red cells. The fetal and adult red cells also differ by one of their surface antigens: i (fetal) vs I (adult).

Table 1: Features of Fetal Versus Adult Erythroid Cell Differentiation

	Fetal rbc	Adult rbc
Hemoglobin type	Hb F	Hb A
Hemoglobin F subtypes gly:ala ratio	3:1	2:3
Membrane antigen	i	I
Carbonic anhydrase B	0	+
Hemoglobin A_2	0	+

In many cases of leukemia there is reactivation of fetal hemoglobin synthesis. This phenomenon is not peculiar to any specific type of leukemia but has been observed in virtually all types of leukemia: erythroleukemia, acute lymphoid leukemia and acute or chronic myeloid leukemia. In most of these cases however, the finding of increased Hb F is variable and the levels observed are usually low (2–15 %). In erythroleukemia elevations of Hb F are more common and the levels can be quite high (up to 60 %) (3).

In one form of leukemia, juvenile chronic myeloid leukemia (CML), striking elevations of Hb F are almost invariable. The Hb F level usually increases as the disease progresses and can attain up to 70 % of the total Hb. This condition is associated with absence of the Ph_1 chromosome and differs in its clinical course from the adult type of chronic myeloid leukemia (3). The fetal Hb in this condition almost invariably is of the true fetal type with respect to its content of $^{G}\gamma$ and $^{A}\gamma$ chains ($^{G/A}$ or gly/ala ratio). As the disease progresses the red cells also gradually acquire other fetal characteristics: increase in i antigen, and diminution of I antigen, Hb A_2 and carbonic anhydrase B. In this condition, there is therefore an apparent total reversion from adult to fetal protein synthesis. In rare cases of erythroleukemia in infants, a similar total reversion to fetal protein synthesis has been observed (4).

In most other cases of leukemia however, the synthesis of Hb F is less striking (2–15 % of total Hb) and when it occurs, the Hb F is heterogeneously distributed among the red cells: it is usually limited to a small proportion or clone of red cells. In these cases there is usually no other evidence of fetal red cell protein synthesis as in juvenile CML. The significance of the phenomenon is uncertain. Reactivation of Hb F synthesis is also seen in a number of other medical conditions, usually associated with some bone marrow stress and hyperplasia. It is possible that these phenomena cause the nonspecific proliferation of usually dormant fetal clones of red cells. On the other hand, the expression of fetal globin genes may be a consequence of the neoplastic process: other neoplastic processes are sometimes associated with the synthesis of other fetal proteins, such as carcinoembryonic antigen (CEA) in colonic carcinoma and α-feto protein in hepatoma. The precise relationship of these events to the malignant process remains to be elucidated.

Another observation has been made which may have relevance to stem cell regeneration and proliferation following chemotherapy for leukemia. Sheridan, *et al* (4) studied hemoglobin synthesis in a number of patients undergoing chemotherapy

for acute myelogenous leukemia. In most cases they observed a burst of fetal hemoglobin synthesis at about 90 days after start of therapy usually following a period of marrow hypoplasia which preceded a remission. Peak levels of 13 % were observed. The Hb F was distributed in a distinct cell line and the levels decreased to normal during the period of remission. The $^{G/A}$ ratio of the Hb F produced was usually of the true fetal type rather than of the adult type (4), but other features of fetal protein synthesis were not observed. Similar observations have been made in the early stage of bone marrow regeneration following bone marrow transplantation for aplastic anemia or leukemia (4 a). It is conceivable that stem cells after suppression of growth and during regeneration go through a cycle of producing committed stem cells which proliferate and differentiate as fetal cells. These observations will no doubt serve as the basis for further studies on the mechanisms and control of stem cell growth and proliferation.

III. Hemoglobin synthesis during erythroid cell maturation

1. Introduction

In order to delineate mechanisms involved in the control of gene expression during normal cell development, we studied a model system consisting of erythroid cells at various stages of differentiation. Erythroid precursor cells were isolated from the spleens of anemic mice, then fractionated by velocity sedimentation into relatively pure populations of cells at different morphologic stages of maturation. These cells were then analyzed before and after overnight culture in the presence of erythropoietin for heme synthesis, globin synthesis and globin mRNA content by RNA-DNA hybridization assays using as probes the radioactive DNA copy (cDNA) synthesized from reticulocyte globin mRNA by viral reverse transcriptase. The results demonstrated that heme synthesis is maximal at an earlier stage of maturation than hemoglobin synthesis, indicating a certain degree of asynchronism between heme and globin synthesis during erythroid cell maturation. The least mature cells had a low but substantial level of globin mRNA indicating a greater degree of biochemical differentiation than otherwise suggested by the cells' morphological appearance and very low level of hemoglobin synthesis. After culture overnight with erythropoietin, the globin mRNA content of these cells increased three- to five-fold, to levels found in the more mature erythroid precursor cells. These results indicate that the major control of globin gene expression in this system is probably at the transcriptional level, but some degree of translational control may be operative in the early stages of differentiation.

2. Materials and Methods

Hemolytic anemia was induced in virgin female CD_1 mice (Charles River Breeding Labs), 18–24 grams in weight, by intraperitoneal injections of phenylhydrazine, 30 mg per kg, on days 0, 1 and 3. The spleens were removed on day 4, minced in phosphate buffered saline – 15 % fetal calf serum, forced through stainless steel mesh and filtered through 35 micron Nitex cloth. The more mature erythroid cells were lysed with antibody prepared against adult red cells, according to the method of Borsook *et al,* (5), and Cantor *et al,* (6). The cells were

then refiltered through Nitex cloth and separated as a function of size by the velocity sedimentation technique (7, 8) in a Staput Cell Separator with an 18 cm diameter bowl. Approximately 7 x 10^8 cells were loaded in one hour and allowed to settle for 3 hours. After the cone volume was removed, 30 ml fractions were collected and the cells were pelleted at 300 x g. Fractions containing similar morphologic classes of cells were combined into larger pools to provide sufficient material for assay of mRNA and for short-term culture.

Replicate aliquots of cells were suspended in modified McCoy's 5A medium containing 15 % fetal calf serum, penicillin (0.1 units/ml), streptomycin (0.1 µg/ml) and human urinary erythropoietin (0.2 units/ml) at cell concentrations of about 5 x 10^6 cells/ml. Cells were cultured for 16 hours at 37 °C in a humidified atmosphere with 5 % CO_2. In one experiment cells from fraction I were cultured in the presence of 100 µCi/ml of ^{3}H-uridine, (46.2 Ci/m mole) [New England Nuclear Corp.].

Total cellular RNA was extracted from the various velocity sedimentation cell fractions by SDS-phenol-chloroform-isopropyl alcohol extraction at pH 9.0 (9). The cells labeled with ^{3}H-uridine were washed and lysed by homogenization in 0.1 M Tris, pH 7.5, 0.03 M KCl, 0.002 M $MgCl_2$ containing 1 % Triton X 100 and 50 µg/ml of Dextran 70 (McGraw Laboratories). The nuclei were sedimented at 100 x g and RNA was prepared from the supernatant cytoplasm by SDS-phenol-chloroform extraction. The ethanol precipitated RNA was then fractionated by oligo (dT) cellulose column chromatography (9). The RNA initially bound to the column and subsequently eluted by 10 mM Tris HCl, pH 7.5, was ethanol precipitated in the presence of 50 µg of *E. coli* tRNA. It represented 8 % of the initial total cpm in the cytoplasmic RNA.

Mouse reticulocyte RNA was prepared by detergent-phenol-cresol extraction of membrane-free reticulocyte lysates (10), and fractionated by sucrose gradient centrifugation; the RNA sedimenting between 4S and 18S RNA served as partially purified reticulocyte globin mRNA. Further purification of the globin mRNA was achieved by oligo (dT) cellulose chromatography of the sucrose gradient mRNA fraction. The RNA initially bound and then eluted from the oligo(dT)-cellulose was labeled with ^{125}I by Dr. Wolf Prensky (11).

RNA-dependent DNA polymerase was purified from avian myeloblastosis virus by the method of Verma and Baltimore (12). In some preparations, further purification of the enzyme by phosphocellulose chromatography was omitted. ^{3}H-labeled globin cDNA was synthesized from the sucrose gradient purified reticulocyte globin mRNA as previously described (13, 14). cDNA for DNA excess hybridization was synthesized with the following components: α-^{32}P-TTP 22.5 µCi/ml (116 Ci/mmole); TTP, 0.1 mM; dCTP, dATP, dGTP, 0.5 mM; Tris pH 8.3, 50 mM; Mg acetate, 6 mM; NaCl, 60 mM; dithiothreitol, 8 mM; actinomycin D, 50 µg/ml; globin mRNA, 10 µg/ml; RNA-dependent DNA polymerase, 100 µl/ml; and oligo (dT_{12-18}), 2µg/ml. RNA saturation hybridization was then accomplished by incubating a fixed amount of labeled cDNA with varying amounts of total RNA from the different cell fractions for 40 hours at 70 °C, in 0.2 M sodium phosphate, pH 6.8, and 0.5 % SDS (14, 15). Percent hybridization was then determined after digestion of the residual nonhybridized cDNA with the S_1 nuclease of Aspergillus oryzae (14, 15). DNA excess hybridization was performed in 5 µl

of 0.2 M sodium phosphate, pH 6.8 and 0.5 % SDS containing 200–400 cpm of either ^{3}H- or ^{125}I-labeled RNA. Various amounts of ^{32}P-labeled mouse globin cDNA were added to the reaction mixtures which were incubated for 40 hours at 70 °C, in sealed, siliconized, disposable 5 µl micropipettes. After incubation, the reaction mixture was diluted into 2 ml of 2 x SSC (0.3 M NaCl, 0.03 M Na citrate) and incubated for 30 minutes at 37 °C in the presence of 20 µg/ml of boiled pancreatic RNase. Yeast RNA was added to a final concentration of 0.4 mg/ml and TCA added to a final concentration of 10 %. The TCA precipitable radioactivity, collected on Millipore filters was assayed in a Beckman liquid scintillation counter and correction made vor ^{32}P counts.

3. Results

Erythroid precursor cells at different stages of differentiation were obtained from the spleens of mice with phenylhydrazine-induced hemolytic anemia, by immune hemolysis of the more mature cells followed by separation according size by the velocity sedimentation technique. The differential counts of erythroid cells in the three cell fractions examined are listed in Table 2. Fraction I (120 ml)

Table 2: Differential Counts of Erythroid Cells in Velocity Sedimentation Fractions Before and After Culture with Erythropoietin

Velocity Sedimentation Fraction	Hours in Culture	Erythropoietin	Pronormoblasts	Basophilic Normoblasts	Polychromatophilic Normoblasts	Orthochromatophilic Normoblasts	Enucleated RBCs
			%*	%*	%*	%*	%*
I	0	0	73.3	17.8	7.7	1.0	
	16	+	18.5	35.8	24.3	17.6	5.5
	16	–	1.6	7.0	24.6	48.9	17.9
III	0	0	34.1	30.4	31.3	4.3	
	16	+	1.9	14.6	28.6	34.0	20.3
V	0	0	4.4	25.0	48.7	21.0	
	16	+	0.0	0.9	21.3	56.8	21.3

* Percentage of all erythroid cells. Slides were prepared by cytocentrifugation, stained with benzidine-Wright-Giemsa, and differential cell counts performed on 400 cells using a modification of criteria outlined in Reference no. 5.

contained primarily pronormoblasts 73.3 %), the earliest recognizable erythroid precursor, and basophilic normoblasts (17.8 %). Only 8.7 % of the cells contained demonstrable hemoglobin as evidenced by positive staining with benzidine. The more slowly sedimenting fractions (60 ml each) contained progressively smaller, more mature cells. Fraction III contained approximately equal numbers of pronormoblasts, basophilic normoblasts and polychromatophilic normoblasts, while fraction V consisted primarily of poly- and orthochromatophilic normoblasts. As

shown in Table 2, cells from these fractions underwent progressive maturation when cultured for 16 hours in the presence of erythropoietin. Concomitantly, there was a 39–60 % rise in cell number leading to a substantial increase in the absolute numbers of the more mature erythroid cells in each cell population cultured. Cells cultured without erythropoietin differentiated but did not proliferate. Cells from the velocity sedimentation fractions actively synthesized heme and hemoglobin and these functions were also erythropoietin-responsive. The results of heme and globin synthesis by these cells have been reported in detail elsewhere (16). In summary, the results show that, in these cells, heme synthesis is maximal at an earlier stage of differentiation than hemoglobin synthesis indicating a certain degree of asynchronism between heme and hemoglobin synthesis during erythroid cell maturation. These results are summarized in Figure 2. The results of the latter studies

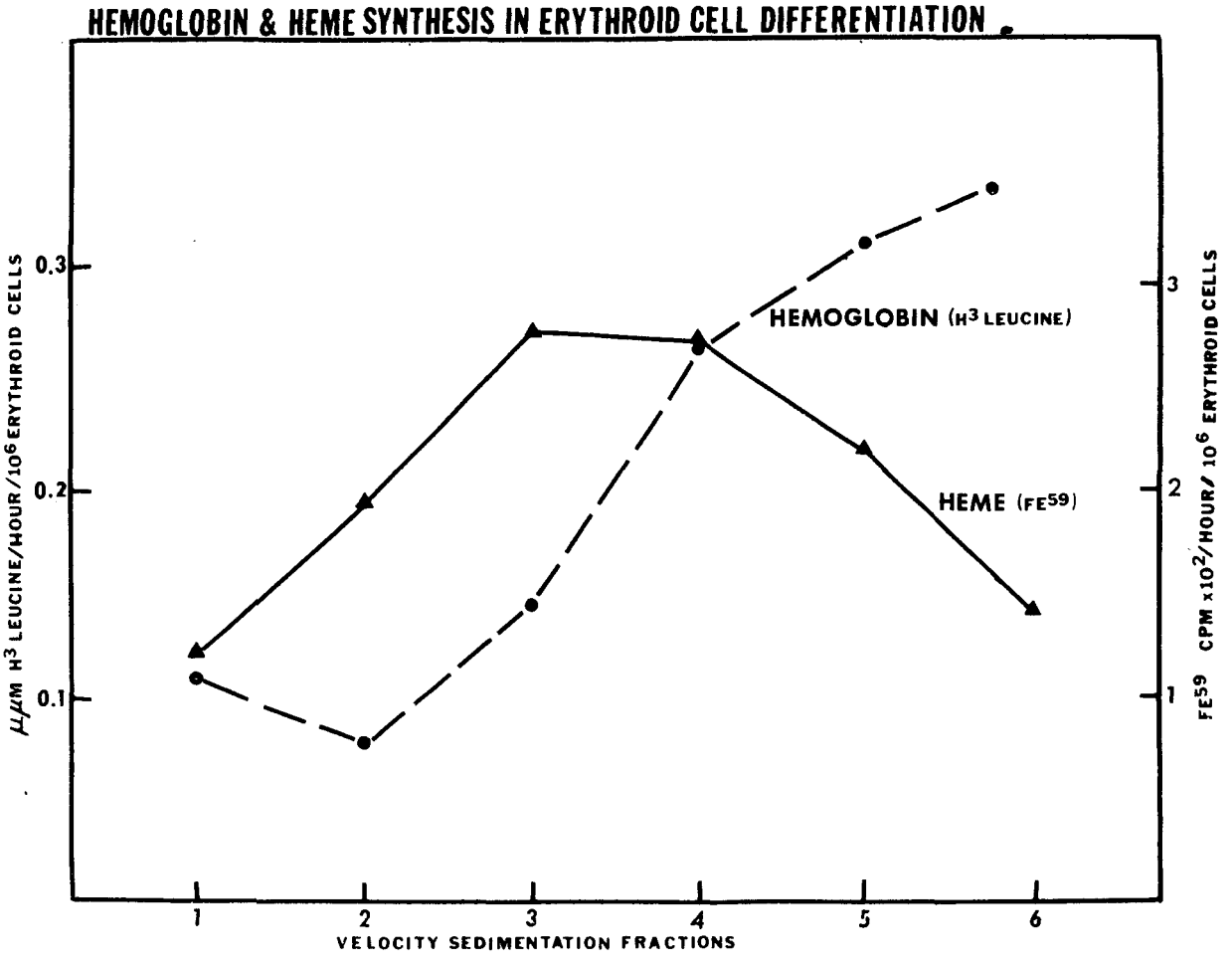

Fig. 2: Relative rates of heme and globin synthesis in the erythroid cells of the various velocity sedimentation cell fractions. The detailed results have been previously published (16) and the figure represents a summary of these results, which indicate a degree of asynchrony between heme and globin synthesis during erythroid cell maturation.

also suggested that erythropoietin is capable of stimulating biochemical differentiation of erythroid precursor cells *in vitro* (16).

Globin mRNA content of the cells was assayed immediately after velocity sedimentation and after culture of replicate samples in the presence of erythropoietin.

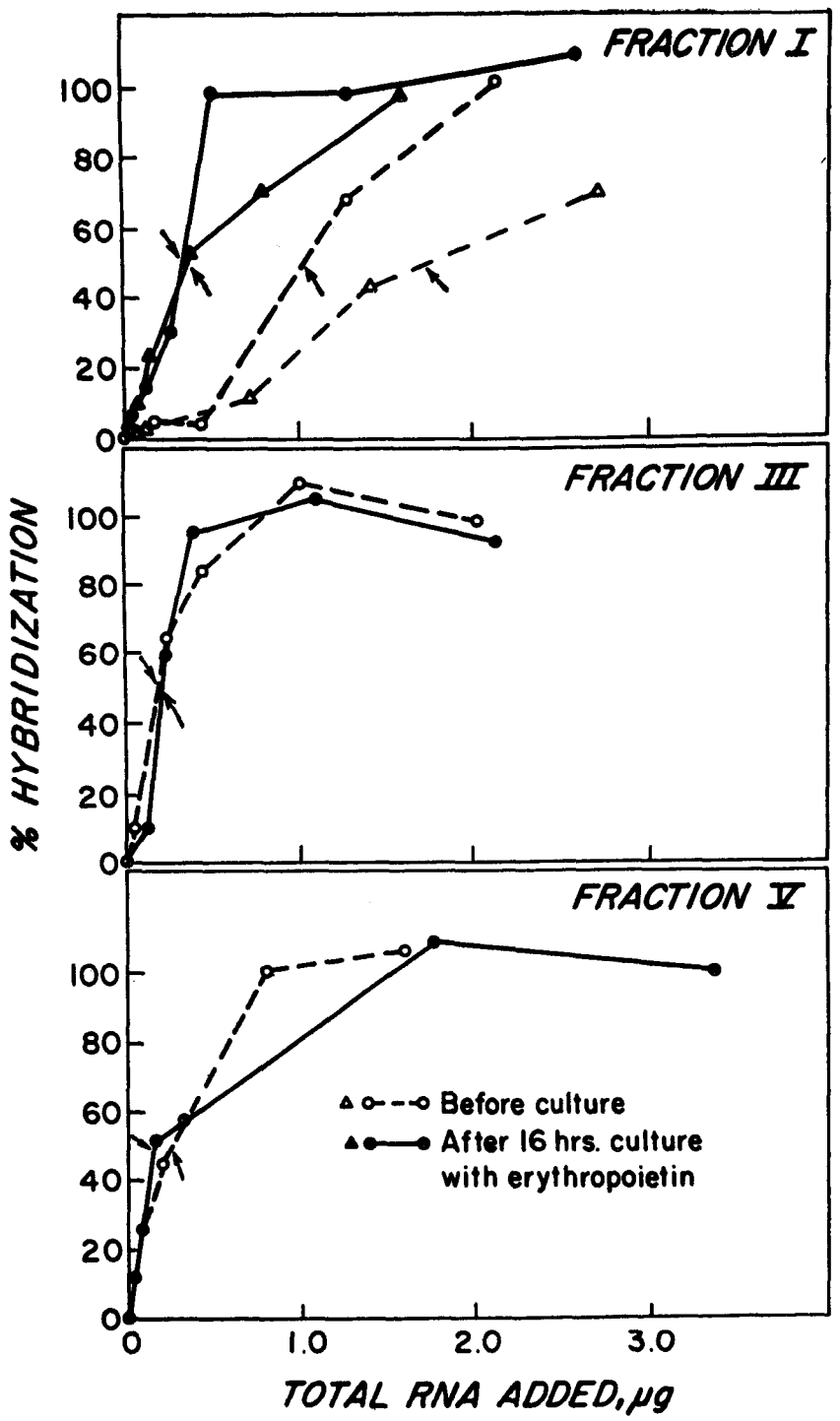

Fig. 3: Hybridization saturation curves using a constant amount of cDNA and increasing amounts of total cellular RNA extracted from velocity sedimentation Fractions I, III, V. Arrows designate 50 % hybridization. The details of the hybridization reaction and its assay are described in Methods. In Fraction I, RNA for the curve (0) was extracted from cells with the differential count listed in Table 2; for the curve (△) the RNA was extracted from a cell population containing only 2.0 % benzidine positive cells (81.5 % pronormoblasts, 16.5 % basophilic normoblasts, 2 % polychromatophilic normoblasts).

Figure 3 shows a series of hybridization-saturation curves using a constant amount of labeled cDNA and increasing amounts of total cellular RNA extracted from the cells in fractions I, III and V. Total cellular RNA varied from about 1.8

A_{260} units per 10^7 cells in fraction I to 1.1 A_{260} units in fraction V. The arrows designate 50 % hybridization. The curve for the most immature cells (fraction I) indicates that 50 % hybridization required 5-fold more RNA than in the more mature fractions III and V; i. e., the proportion of the total RNA that is globin mRNA is 5-fold less in the immature as compared to the mature cells. After overnight incubation of the cells with erythropoietin, the proportion of total RNA that was globin mRNA increased markedly in fraction I, approaching the levels found initially in the more mature fractions. However, no significant change was observed in fractions III and V.

Since globin mRNA levels increased after culture with erythropoietin only in fraction I, an additional experiment was performed with cells from this fraction cultured in the presence or absence of erythropoietin (Figure 4). The 50 % hybrid-

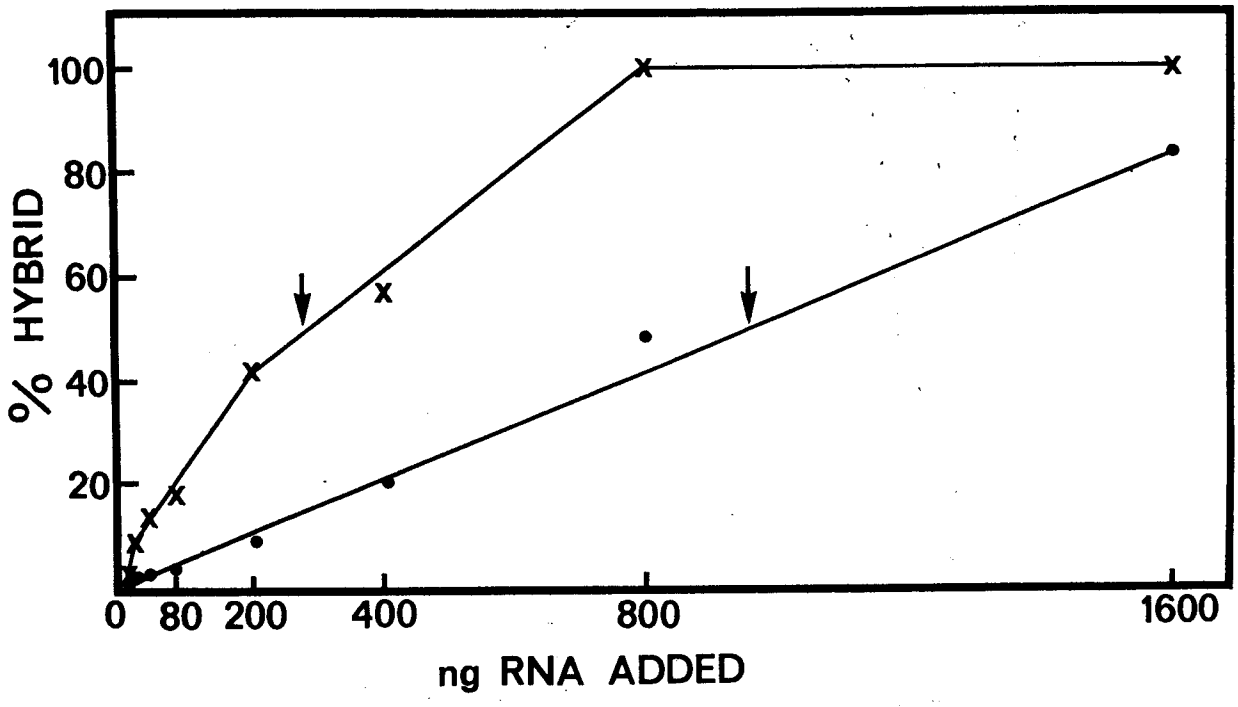

Fig. 4: Hybridization saturation curves using ^{32}P-labeled cDNA and increasing amounts of total cellular RNA from cells of Fraction I cultured in the presence (x) or absence (●) of erythropoietin. Arrows designate 50 % hybridization. The saturation curve for the cells prior to culture was similar to that for cells cultured without erythropoietin and is not presented.

ization values in this experiment indicate that about 3.5 times more globin mRNA was present in the cells cultured with erythropoietin. The level of globin mRNA after culture without erythropoietin was similar to that observed in the same cells prior to culture.

In one experiment the amounts of antiserum and complement used for immune lysis were titered so as to remove nearly all benzidine positive cells (Fig. 3). After velocity sedimentation fraction I contained only 2.0 % benzidine positive polychromatophilic normoblasts. Although the point of 50 % hybridization was achieved with approximately 1.5-fold more added RNA than in previous experiments, the hybridization curve still demonstrated significant levels of globin mRNA.

The quantity of globin mRNA present as a percent of the total cellular RNA was determined by reference to a standard curve for purified mouse globin mRNA hybridized to the same cDNA used in previous studies (14). Levels of mRNA observed in the most immature cells (fraction I) were about 0.008–0.013 % of total cellular RNA and increased to 0.05–0.075 % in more mature precursors (Table 3).

Table 3: Amounts of Globin mRNA in Velocity Sedimentation Fractions Before and After Culture with Erythropoietin

Velocity sedimentation fraction	Hours in culture	Expt. no.:	RNA hybridized to cDNA as % of cell RNA* 1	2	3	4
I	0		0.013	0.009	0.012	0.0083
	16		0.05	0.043	–	–
III	0		0.075	–	0.05	–
	16		0.075	–	–	–
V	0		0.075	–	–	–
	16		0.075	–	–	–

* See text for calculation of values.

Similar levels have been observed by Harrison *et al* in studies of fetal erythroid cells (17). On culture with erythropoietin, globin mRNA increased 4-5-fold in fraction I, but remained constant in fraction III and in fraction V. The apparent stability of globin mRNA in the more mature fractions was associated with morphologic maturation to the point that large numbers of orthochromatophilic normoblasts and enucleated RBC's were now present (Table 2).

To demonstrate definitively *de novo* synthesis of globin mRNA, cells from fraction I were cultured in the presence of ^{3}H-uridine and erythropoietin for 16 hours. The poly(A) containing RNA fraction was then isolated by oligo(dT) cellulose chromatography of the total cellular RNA. Approximately 5 % to 8 % of the ^{3}H-labeled RNA was initally bound and subsequently eluted from the oligo(dT) cellulose column. The level of globin mRNA in the poly(A) containing RNA was estimated by hybridization to varying amounts of ^{32}P-labeled mouse globin cDNA. The fraction of ^{3}H-labeled material in hybrid form was determined by resistance of the hybrid to digestion with RNase. Mouse globin mRNA labeled with ^{125}I *in vitro* was also hybridized to mouse globin cDNA to serve as a control. The results are shown in Table 4. At least 65 % of the ^{125}I mRNA could be protected from RNase digestion by the globin cDNA, indicating that the cDNA contains at least 65 % of the sequences of the globin mRNA molecule. Protection of ^{3}H-labeled RNA was about 50 % suggesting that at least 50 % of the stable poly(A) containing cytoplasmic RNA synthesized by erythroid cells of fraction I during the 16 hour incubation period was specifically globin mRNA.

Table 4: DNA Excess Hybridization of Radioactive RNA

RNA added	ng cDNA added	cpm RNA Hybridized	% Hybridization
A) ^{3}H-uridine labeled poly A-containing RNA	0	39.4	–
	0.5	52.7	10
	1.0	60.8	16.5
	2.5	68.9	23
	5	99.2	46.5
	6.6	105.0	51
B) ^{125}I-labeled Mouse Reticulocyte 10S RNA	0	33.3	–
	0.01	44.3	4
	0.025	57.7	7
	0.05	102.5	18
	0.1	192.0	40.5
	0.25	185.5	39
	0.5	244.2	54
	1.0	287.8	65

A fixed amount of radioactive RNA was hybridized to the indicated amounts of cDNA. The quantity of cDNA was calculated on the basis of the specific activity of α-^{32}P-TTP incorporated into the cDNA. The details of the hybridization reaction and its assay are outlined in Methods.

A) Reaction mixtures contained 149 cpm of ^{3}H-labeled RNA derived from the cytoplasm of mouse spleen cells of Fraction I incubated overnight in the presence of erythropoietin and ^{3}H-uridine. Only the poly A-containing RNA (bound to oligo-(dT)-cellulose) was utilized.

B) Reaction mixture contained 418 cpm of ^{125}I-labeled mouse reticulocyte 10S globin mRNA, which had been purified by oligo(dT)-cellulose chromatography prior to iodination. Background was 20 cpm.

4. Discussion

The purpose of this study was to investigate the development of globin mRNA in erythroid cells in an experimental system in which cell differentiation can be readily assessed *in vitro* and which is responsive to erythropoietin. The cell separation methods employed yielded a population of immature cells which contained low levels of globin mRNA but in which the level of globin mRNA increased substantially during the course of incubation with erythropoietin.

The most immature erythroid cell population isolated by the cell separation procedures was comprised primarily of pronormoblasts, the earliest recognizable erythroid cell, whereas the most mature cell population contained predominantly well hemoglobinized precursors. Small amounts of globin mRNA were already detectable in the earliest cell fraction, but levels were higher by a factor of 4–5 in the more mature nucleated erythroid cells. This suggested that the latter must have synthesized significant amounts of globin mRNA as they matured *in vivo*. A similar correlation between globin mRNA levels and progression of erythroid

cell maturation has been demonstrated with erythroid cells from the spleens of mice after phenylhydrazine treatment, using a cell-free translation assay (18, 19).

New synthesis of mRNA during erythroid cell maturation was demonstrated more directly in the short-term culture experiments. As the cells in fraction I underwent morphological differentiation *in vitro,* there was a substantial increase in the content of globin mRNA. This change was found only when the cells were cultured in the presence of erythropoietin. Newly synthesized ^{3}H-labeled globin mRNA could also be demonstrated in the ^{3}H-uridine labeled RNA of these cultured cells, using RNA-cDNA hybridization (Table 4). On the other hand, the levels of globin mRNA remained stable with culture of the more mature cell fractions. Erythropoietin has also been shown to increase the globin mRNA content of fetal liver erythroid cells, as measured by both translational (20) and hybridization assays (21).

The precise point in the sequence of erythroid differentiation at which globin mRNA synthesis is initiated is yet to be determined. Terada *et al* have studied this problem by using translation in a cell-free system to assay globin mRNA in primitive erythroid cells from mouse fetal liver (20). These authors found negligible levels of functional globin mRNA in cell populations consisting of about 30 % proerythroblasts, 70 % basophilic erythroblasts and less than 7 % benzidine positive cells. These cells required culture with erythropoietin for at least ten hours before their RNA developed the capacity to direct globin synthesis. Ramirez *et al* (21) using hybridization techniques have found only negligible amounts of globin mRNA in very early fetal erythroid cells, but this level increased 250-fold after culture for 22 hours in the presence of erythropoietin. Using similar cDNA:RNA hybridization assays, we have consistently detected small but significant levels of globin mRNA in the very early erythroid cell population of fraction I.

The low levels of globin mRNA already present in this youngest cell population may have been contributed in part by the nearly 9 % benzidine-positive cells that contaminated this fraction (Table 2). However, substantial hybridization was observed in an experiment in which fraction I contained only 2 % benzidine-positive cells (Fig. 3). Thus, early cells apparently did contain much of the globin mRNA found in the RNA of fraction I cells. This result indicates that there is more biochemical differentiation in these cells than is apparent from the morphology of these otherwise very primitive cells. The erythroid cells were obtained from animals with severe anemia and hence had been subjected to high levels of erythropoietin *in vivo.* As suggested by Harrison *et al* (17), erythropoietin may increase the proportion of pronormoblasts containing globin mRNA.

Other findings which lend support to the presence of globin mRNA in immature erythroid cells have been reported by Harrison *et al* (17, 22). These investigators localized globin mRNA by radioautography after *in situ* hybridization to ^{3}H-labeled cDNA and found a small amount of globin mRNA in the cytoplasm of some pronormoblasts and most basophilic normoblasts from 13.5 day fetal liver in the mouse. The radioautography technique permits localization of mRNA in specific cells, but probably is not as reliable as saturation hybridization to quantitate globin mRNA. The same authors also performed conventional hybridization studies (17), the results of which also indicated that immature fetal erythroid cells contain substantial amounts of globin mRNA. These studies in fetal erythroid cells

are therefore very similar to the findings for adult erythroid precursors in the present study. Unexplained are the differences between these results and those which suggest very low levels of globin mRNA in the immature fetal system (20, 21).

The new techniques used in this and other recent studies begin to make it possible to investigate the specific mechanisms controlling the synthesis of globin mRNA. Several groups (23–25) have shown that erythropoietin stimulates the synthesis of several species of RNA in cultures of erythroid precursor cells, but no specific assays were used to identify newly synthesized globin mRNA. In future studies, the use of specific hybridization probes now available for the detection of globin mRNA should lead to a better understanding of the interaction between erythropoietin and the expression of the genes that control globin synthesis.

IV. Summary and Conclusions

We have reviewed erythroid cell differentiation from two points of view: 1) differences between fetal and adult human red cells with particular reference to alterations which can occur in the normal pattern of erythroid cell development during the course of leukemia; 2) beochemical events which occur during erythroid cell maturation, as a model system for the study of the control of gene expression.

During the course of many leukemias there is the synthesis of red cells containing fetal hemoglobin. In most cases this phenomenon is limited to a small population or clone of red cells and probably represents a nonspecific response of the bone marrow to a hematologic stress. However, in juvenile chronic myeloid leukemia and, in rare cases of erythroleukemia, there is a major reversion to fetal erythropoiesis, with progressive increase in fetal hemoglobin levels and synthesis of red cells which contain not only fetal hemoglobin but have a true fetal pattern of protein synthesis affecting proteins other than Hb F, namely Hb A_2, carbonic anhydrase and the membrane antigens i and I. In this case, the fetal erythropoiesis may be a more specific manifestation of the leukemic process and may be related to the phenomenon of fetal protein synthesis (α-fetoprotein of carcinoembryonic antigen) observed in other types of neoplasia.

Further information on the etiology and pathogenesis of abnormal cell proliferation and differentiation in the leukemias can be obtained by the study of experimental systems permitting the investigation of the regulation of gene expression in differentiating mammalian cells. Maturing erythroid cells provide a promsing system for such investigations for many reasons: differentiating erythroid cells can be obtained relatively free of other cell types; a large amount of a well characterized product, hemoglobin, is synthesized; techniques are now available that permit isolation of erythroid precursors at different stages of differentiation (5–8); and finally, highly sensitive methods of measuring globin mRNA levels by DNA-RNA hybridization are currently available (13, 26, 27). We have used such techniques to measure levels of globin mRNA in separated populations of murine erythroid cells at different stages of maturation. These studies demonstrated a correlation between globin mRNA content and degree of morphological maturation. In the least well differentiated cells, however, there appeared to be a disproportionate amount of

mRNA for the level of hemoglobin synthesis in these cells. These results suggest the presence of some translational control of globin mRNA in the early stages of erythroid development, although the major control of globin gene expression in this system seems to be at the transcriptional level. Finally, when the immature erythroid cells were cultured in the presence of erythropoietin, *de novo* synthesis of ^{3}H-uridine labeled globin mRNA was demonstrated by the specific RNA-cDNA hybridization assay. These results clearly demonstrate the utility of this model system and these techniques for the study of the interaction between a specific gene and the factors which regulate or modulate its expression.

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